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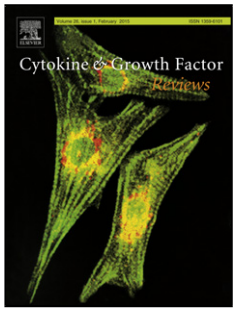

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Vascular endothelial growth factors and placenta growth factor in retinal vasculopathies: current research and future perspectives

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Highlights

► A review of the function of growth factors VEGF-A, VEGF-B and PlGF in normal development and disease.

► The implication of VEGF-A, VEGF-B and PlGF in the pathophysiology and pathogenesis of various ophthalmic disorders.

► A discussion of investigational studies of human vitreous and serum VEGF-A, VEGF-B and PIGF and importance in diagnostic and therapy.

► An analysis of current targeted therapies for retinal diseases.

► A reflection on new promising molecules in ophthalmology.

Abstract

Vision loss due to disease or degeneration of the eye (retina, choroid, retinal veins, or macula) is a leading cause of blindness worldwide. In most cases, vision-threatening ocular diseases are accompanied by abnormal changes in the vasculature of the eye, especially the retina, and these conditions are collectively referred to as retinal vasculopathies. Impaired blood supply or hypoxia stimulates angiogenesis in the vascular and non-vascular sections of the eye, which results in neovascularization, leading to conditions such as diabetic retinopathy or age-related macular degeneration. Studies show that vascular endothelial growth factors: VEGF-A, VEGF-B, and placental growth factor (PlGF) are elevated in these diseases, and hence, these factors could be used as markers for disease prognosis and therapy. In this review, we discuss the function of these growth factors in normal development and disease, with focus on ocular disorders and emphasize the importance of accurately determining their levels in the vitreous and serum of patients for correct diagnosis and therapy.

Keywords: Angiogenesis, neovascularization, ophthalmic disorders, placental growth factor (PIGF), vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor B (VEGF-B)

1. Introduction

Growth factors are naturally occurring molecules, usually proteins or steroids, which stimulate cellular growth, proliferation, and differentiation [1]. Typically, growth factors act as signaling molecules between cells and bind to cell surface receptors to initiate signaling cascades that affect gene expression and cell fate. For example, bone morphogenetic protein induces bone differentiation [2], whereas fibroblast growth factor [3] and vascular endothelial growth factors (VEGF) stimulate angiogenesis, i.e., growth of blood vessels [4,5].

For the last two decades, growth factors are also being extensively used in medicine for treating hematologic, oncologic, and cardiovascular diseases [6,7,8,9]. In particular, the VEGF family of growth factors are finding increasing use in the treatment of cardiac [10], renal [11], and bone-related diseases [12] as agonists and in ophthalmic diseases [13,14] and cancer as antagonists [15]. Angiogenesis plays important roles in normal and pathological proliferative processes. It is involved in normal growth, wound healing [16,17,18] as well as in tumor growth and metastases [19], which makes regulators of angiogenesis critical biomedical molecules.

The VEGF family consists of seven secreted dimeric proteins, namely, VEGF-A (or VEGF), B, C, D, E (or viral VEGF), F (snake venom VEGF) and the placental growth factor (PlGF) [20]. VEGF-A has several isoforms that arise via alternative splicing of the eight-exon *VEGF-A* gene. All VEGFs function by binding to cell surface-bound tyrosine kinase receptors called vascular endothelial growth factor receptors (VEGFRs), causing them to dimerize and be activated through transphosphorylation, albeit with different specificities. VEGF ligands bind to three main transmembrane endothelial receptors namely, VEGFR-1, VEGFR-2, and VEGFR-3 [21]. In addition, neuropilins, neuropilin 1 (NRP-1), and neuropilin 2 (NRP-2) provide co-receptor function in endothelial cells [22]. Among the growth factors of the VEGF family, VEGF-A is well-studied and PlGF less studied in terms of function and

clinical application, whereas the physiological role of VEGF-B was ambiguous until recently and is just beginning to be understood. Nonetheless, the effect of these three factors in ophthalmic development and diseases is relatively less investigated. In this review, we would first discuss the roles of VEGF-A, VEGF-B, and PlGF in general development, followed by discussion of their emerging functions in ophthalmic disorders, as well as targeted molecules.

2. Vascular endothelial growth factors in development and disease

2.1. VEGF-A

VEGF-A, which signals via VEGFR-1 and 2 and NRP-1 and 2 [23,24], is essential for vascular development. Mice lacking any of the VEGF allele die during embryogenesis due to impaired angiogenesis [25]. Alternative exon splicing produces seven VEGF-A isoforms: $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. Each isoform is characterized by the respective number of amino acids after cleavage of the signal sequence variants [26]. Initial in vitro studies showed that capillary endothelial cells proliferate and form tubular structures in the presence of VEGF-A [27]. VEGF-A expression is downregulated after embryogenesis [28], whereas it is upregulated during physiological and pathological angiogenesis [29] and also during exercise and muscle contraction [30]. Increased blood flow during exercise along with VEGF-A also stimulates the production of its receptors, which initiates a massive signaling cascade leading to the production of nitric oxide (for vessel permeability), FGF (for cell proliferation), and ICAM/VCAM/matrix metalloproteases (for migration) all of which contribute to the formation of new blood vessels. In addition, VEGF and VEGFR-1 are upregulated in hypoxic conditions via hypoxia inducible factor (HIF) 1-α-dependent [31] and independent pathways [32], highlighting the close relationship between blood oxygen levels, angiogenesis, and tissue metabolism. Similarly, the levels of VEGF-A and its corresponding receptors arise immediately after a traumatic injury to the central nervous system and decline with time, which corresponds to

endogenous post-injury revascularization [33].

Rheumatoid arthritis (RA) is an autoimmune disease, and synovial angiogenesis is critical for inflammation and immune activation associated with RA pathogenesis. A recent study showed that serum VEGF-A levels and VEGF-A polymorphisms were strongly associated with incidence of RA in the Polish population [34]. Cancer cells require high nutrient and oxygen supply for rapid proliferation and dissemination (metastasis), which necessitates *de novo* angiogenesis inside the tumor; thus, cancer may be categorized as an "angiogenic" disease. Studies have shown that VEGF-A overexpression is implicated with poor prognosis of breast cancer, which acts as an angiogenic switch that induces metastasis [35]. Similarly, VEGF-A has been found to be upregulated in hepatic, gastric, pancreatic, ovarian, bladder, colorectal, myeloid, and thyroid cancers and medulloblastoma [7]. Thus, VEGF-A is a good candidate for anti-cancer treatment, and an anti-VEGF-A monoclonal antibody called bevacizumab was clinically approved for cancer treatment in 2004. VEGF-A is also involved in renal and pulmonary disorders. Patients with pulmonary emphysema have decreased VEGF-A levels in pulmonary arteries [36]. VEGF-A is also a biomarker of asthma and compulsive obstructive airway disease [37]. In contrast, increased VEGF-A expression is associated with glomerular hypertrophy and proteinuria [38] although physiological levels of VEGF-A are required for renal development and maintenance of glomerular capillary structure.

2.2. VEGF-B

VEGF-B is highly related to VEGF-A, although it signals via the VEGFR-1 receptor unlike that of VEGF-A, which uses both VEGFR-1 and 2, thereby competing with VEGF-A for VEGFR-1 binding [39]. VEGF-B also binds with NRP-1 [39]. Despite similarity in structure, the physiological role of VEGF-B is ambiguous. VEGF-B has two isoforms: VEGF-B_{167} and VEGF-B_{186}. Both VEGF-B isoforms can form heterodimers when co-expressed with VEGF-

A_{165} in cells; however, whether heterodimers exist in nature is not known. Despite being cell-bound, the VEGF-A_{165}/VEGF-B_{167} heterodimers can be freely secreted by endothelial cells. The balance between homo- and heterodimers can affect VEGF-A signaling if heterodimers are formed [40].

VEGF-B possesses low angiogenic potential; hence did not induce vessel formation or sprouting when delivered in muscle or periadventitial tissue [41], whereas transgenic overexpression of VEGF-B minimally increased vasculature. In contrast, VEGF-B overexpression was reported to potentiate and not initiate angiogenesis in endothelial cells of transgenic mice [42]. VEGF-B was therefore considered as a survival factor of endothelial cells that regulated the expression of pro-survival genes via VEGFR-1 and NRP-1 signaling [43].

Unlike VEGF-A, VEGF-B is not induced by hypoxia, other growth factors, cytokines, hormones, or oncogenes [44,45]. Although a recent study has reported that hypoxia induces VEGF-B in the retina [46]. However, VEGF-B is expressed in a wide range of tissues; being most abundant in tissues with high metabolic activity, such as the myocardium, skeletal muscle, vascular smooth muscle, brown adipose tissue, kidney, brain, and parietal cells of the stomach [47,48]. This indicates a role of VEGF-B in coordinating angiogenesis with metabolism. A study demonstrated that VEGF-B is highly expressed in metabolically active tissues, such as the heart and skeletal muscle, suggesting its function in maintaining oxidative metabolic and contractile function in these tissues [49]. VEGF-B is expressed on the cell surface of cardiomyocytes, which are released for downstream signaling after cleavage with endothelial cell-secreted heparanase. The bidirectional interaction between endothelial cells and cardiomyocytes could provide the diabetic heart protection against cell death and may be a critical tool for delaying or preventing cardiomyopathy. However, VEGF-B and heparanase production decline under sustained hyperglycemic conditions and VEGF-B signaling declines

albeit upregulation of VEGFR-1, which results in diabetic cardiomyopathy [49]. Another study identified VEGF-B to be a coronary growth factor in rats where it induced cardiac hypertrophy via the endothelium [50]. VEGF-B also induced angiogenesis and arteriogenesis in myocardium of patients via VEGFR-1 and NRP-1 signaling [51]. Moreover, VEGF-B selectively promotes angiogenesis in ischemic myocardium. Several studies have shown that VEGF-B has a specific role in the revascularization of ischemic myocardium in different disease models of mice, pigs, and rabbits. Therefore, VEGF-B might have a cardioprotector effect and might harbor therapeutic potential for ischemic heart diseases [42,48].

Studies in *VEGF-B* knockout mice show that *VEGF-B* is dispensable for embryonic growth and survival, unlike *VEGF-A* knockouts that die during embryogenesis. However, the hearts of the *VEGF-B* knockout mice were smaller and displayed vascular dysfunction after coronary occlusion and impaired recovery from experimentally-induced myocardial ischemia, indicating a role of VEGF-B in coronary vasculature development [52].

Diabetic kidney disease (DKD) is a severe renal disease that is characterized by defects in glomerular filtration, proteinuria, and steatosis. DKD tissues show high VEGF-B expression. Since VEGF-B controls muscle lipid accumulation through regulation of endothelial fatty acid transport [53], therefore therapeutic reduction in VEGF-B levels ameliorates symptoms of DKD, such as renal lipotoxicity and insulin insensitivity [54].

VEGF-B also has a crucial role in neuroprotection. VEGF-B-deficient mice showed impaired recovery from cerebral ischemic injury and neurogenesis was seen to be stimulated in adult mice on administration of VEGF-B [55]. VEGF-B is also required for nerve regeneration, sensory recovery, and trophic functions of injured corneal peripheral nerves, but is not present in a mouse model having no nerve injury [56]. In addition, VEGF-B was shown to increase angiogenesis in a mouse model of arthritis as *Vegfb-/-* mice exhibited decrease in inflammation-associated synovial angiogenesis [57].

The role of VEGF-B in cancer remains unclear. However, Yang and colleagues demonstrated that VEGF-B promotes cancer metastasis through remodeling of tumor microvasculature and a VEGF-A–independent mechanism [58]. The function of VEGF-B is multifaceted, thus it was considered by Li and co-workers as a survival factor rather than an angiogenic molecule [59].

2.3. PlGF

PlGF is a pleiotropic factor that affects different cell types and regulates various biological processes via signaling through VEGFR-1. PlGF has four splice variants that are generated via alternative splicing: PlGF-1 ($PlGF_{131}$), PlGF-2 ($PlGF_{152}$), PlGF-3 ($PlGF_{203}$), and PlGF-4 ($PlGF_{224}$) [60]. The PlGF-2 and PlGF-4 isoforms also bind to neuropilins. PlGF can also form heterodimers with VEGF-A [61]. One of the primary functions of PlGF is regulation of vessel growth and maturation, and therefore, this cytokine is associated with pro-angiogenic activities similar to VEGF-A. PlGF recruits myeloid progenitors to growing sprouts and collateral vessels; it attracts macrophages, which release angiogenic and lymphangiogenic factors. In addition, PlGF regulates ossification, wound healing, retinal pigment cell chemotaxis, and survival of cortical neurons, etc. [62] PlGF overexpression in murine epidermal cells elicited severe inflammatory response associated with pronounced edema, inflammatory cell infiltration, and vascular enlargement indicating direct role of PlGF in cutaneous inflammatory response [63].

Sandro De Falco and colleagues described the role of PlGF in cardiovascular diseases and suggested three major functions for PlGF in the cardiovascular system: 1) myocardial angiogenesis, 2) mediating macrophage chemotaxis, 3) selective action in modulating pathological rather than physiological vascular development, which makes this protein an excellent candidate for therapeutically modulated angiogenesis [64].

PlGF levels are low in normal adult tissues; however, it was upregulated in 4 out of 16 menangioma tumors, whereas VEGF-A level was upregulated in 3 out of 16 samples. There was no significant correlation between PlGF and VEGF-A expression levels. VEGF-B was uniformly expressed in all tumor samples. In a PlGF-positive tumor sample, immunoreactive VEGFR-1 and VEGFR-2 were detected in endothelial cells of the blood vessels and PlGF was detected in most tumor capillaries. Thus, PlGF might be yet another marker for tumor angiogenesis in human menangiomas [65]. Interestingly, supra physiological levels of PlGF have been documented to inhibit angiogenesis of tumors co-expressing VEGF-A. This possibly occurs because of heterodimerization of PlGF with VEGF-A, which outnumbers the biologically active VEGF-A homodimer [66]. Indeed, Yang et al. demonstrated that PlGF can affect tumor angiogenesis in both positive and negative ways in a VEGF-A-dependent manner. In one tumor model, PlGF remodeled tumor vasculature to a normalized phenotype, whereas ablation of VEGF-A in a PlGF-positive tumor accelerated tumor angiogenesis and growth [67].

3. Vascular endothelial growth factors in ophthalmic diseases

3.1. *VEGF-A*

The eye possesses a special anatomy where completely avascular and highly vascular structures lie in close apposition. Stringent regulation of the balance between vascular growth and quiescence maintains this structure. Vascular growth occurs mainly during embryonic development and is almost absent in the adult eye. Therefore, ophthalmic diseases associated with angiogenesis represent cases where this delicate balance has been disturbed by external conditions, such as hyperglycemia, oxidative stress and other factors (Figure 1) [68].

Vitreous levels of VEGF-A were high in several retinal diseases, such as diabetic retinopathy (DR), age-related macular degeneration (AMD), and retinal vein occlusion (RVO) [69].

Among diseases contributing to ocular anomalies, diabetes has been studied extensively owing to its manifold effects on healthcare [70]. The deleterious effects of diabetes mellitus on macro and microcirculation underline the morbidity and mortality associated with this disease. Research on pathological factors related to diabetes mellitus have highlighted the involvement of VEGF-A in conditions, such as DR, diabetic macular edema (DME) and AMD, which are the major causes of blindness worldwide [68]. DR is accompanied by loss of the retinal barrier function and increased vascular permeability. Several blood-borne proteins might contaminate the vitreous and show an elevated level; therefore, simultaneous testing of protein levels in both serum and vitreous is crucial for correctly interpreting whether the increased vitreal expression is due to upregulation of gene expression or vascular leakage. An accurate interpretation of vitreal pro-angiogenic protein levels is important for the development of correct biomarkers and therapies for retinal diseases [71].

Proliferative DR (PDR) is characterized by progressive loss of retinal capillaries, followed by hypoxia and hypoxia-induced VEGF-A expression, which stimulates neovascularization of the retina, disc, angle, and iris. Several clinical studies have confirmed the correlation between ischemic retinopathies and RVO and VEGF-A levels [69,72]. Elevated VEGF-A levels were observed in the aqueous and vitreous samples of 143 patients with proliferative retinopathies undergoing intra-ocular surgery [73]. On the other hand, laser surgery considerably reduced the intraocular VEGF-A levels [74]. VEGF-A levels were considerably lower in individuals with non-neovascular disease or diabetes without retinopathy. Similar results were reported by Aiello et al. [69], who detected high levels of VEGF-A in 69/136 ocular fluid samples from patients with DR, 29/38 iris samples with neovascularization, and 3 of 4 samples from patients with ischemic occlusion of the central retinal vein, compared to 2 of 31 samples from patients with no neovascular disorders. Other studies showed that advanced glycation end products and decreased anti-oxidant status correlated with DR

pathogenesis via VEGF-A induction [75]. A quantitative proteomic study using the vitreous of patients with PDR and non-PDR and those treated with anti-VEGF-A therapy revealed 230 proteins involved in inflammation, complement activation, cell adhesion, and the coagulation cascade, and apolipoproteins, immunoglobulins, etc. to be overexpressed in PDR than in non-PDR [76]. This reflects the multifactorial nature of DR and suggests new possibilities for developing therapeutics. Elevated levels of various cytokines, such as interleukin- (IL-) 1β, IL-2, IL-4, IL-5, IL-6, IL-10, interferon-γ, tumor necrosis factor-α, and VEGF were observed in the aqueous humor of patients with DR, and the levels increased with the severity of the disease [77].

Neovascularization of the angle leads to neovascular glaucoma (NVG), which is currently being treated with anti-VEGF-A therapy in clinical trials [78] as VEGF-A is a marker for NVG in the aqueous humor and serum of such patients [78]. However, it is noteworthy that VEGF-A expression is observed in both normal and diabetic retina photoreceptor and ganglion cells, indicating a physiological role of this growth factor in ocular angiogenesis [79]. A proteomic analysis showed that VEGF-A was upregulated in AMD [80] and that vitrectomy, followed by retinal photocoagulation, decreased VEGF-A levels [81].

DME is a vision-threatening complication of DR, with a prevalence rate of 20% and 25% in patients with type I and type II diabetes, respectively. DME is caused by macular thickening and cyst formation post retinal-blood barrier breakdown, increased vascular permeability, and fluid accumulation in hyperglycemic condition. Optical coherence tomography (OCT) and enzyme-linked immunosorbent assay (ELISA) of the vitreous of 71 patients with DME revealed a concentration gradient of VEGF-A in DME from the macula to the periphery and from the posterior to the anterior globe [82]. Laser treatment as well as intravitreal anti-angiogenics, such as ranibizumab, aflibercept, and bevacizumab (off-label) have been widely used for the treatment of diseases targeting VEGF-A and are considered as the gold standard

for treatment of angiogenic disorders. Intravitreal corticosteroid administration is also used to reduce VEGF levels and angiogenesis in patients with DME [83]. Moreover, other revolutionary modes of treatment, such as dexamethasone and fluocinolone acetonide corticosteroid implants are being used for the treatment of angiogenic and inflammatory diseases [84].

In addition to DR and AMD, VEGF-A causes multiple age-related eye diseases such as cataracts and neovascular and non-exudative AMD-like pathologies. High VEGF-A levels induce age-related opacifications in the lens, which is accompanied by ERK activation, inflammation, and oxidative damage. Targeting of inflammasome components considerably downregulated VEGF-A-stimulated cataract formation. Elevated VEGF-A also causes choroidal neovascularization [85].

It is noteworthy that VEGF-A is the most studied growth factor till date. However, other growth factors are also involved in these angiogenesis-associated pathologies, such as VEGF-B and PIGF, which are gradually gaining attention in basic and clinical research.

3.2. *VEGF-B*

Previously, the role of VEGF-B in ophthalmic development and diseases was obscure; however, recent studies have shed light on the function of this enigmatic molecule. For example, the overexpression of VEGF-B promoted pathological retinal and choroidal neovascularization and blood-retinal barrier disruption without inflammation, unlike VEGF-A [85]. Thus, VEGF-B could be involved in the progression of DR and AMD in an inflammation-independent way and could therefore be used for developing anti-angiogenic therapies [86]. Interestingly, Reichelt et al. [87] reported that VEGF-B was not required for the development of retinal vasculature under normal conditions or in oxygen-induced retinopathy.

In a study performed by our research group, the measurement of VEGF-B levels in patients with DR and rhegmatogenous retinal detachment revealed that VEGF-B levels were significantly higher (p=0.006) in the vitreous of diabetic patients with ocular disease, and the levels increased in advanced stages of DR [88]. In another study, we estimated VEGF-A and VEGF-B levels using ELISA in the vitreous and serum of patients with proliferative ocular disorders (POD), which included patients with DR, AMD, and retinal vein occlusion (n = 10), and compared it with a control group of patients with non-proliferative ocular disorders (NPOD) (n = 4). Similar to the results of earlier studies, we observed that VEGF-A and B levels were elevated in POD than in NPOD mainly because of DR, and that the serum and vitreous levels of VEGF-A and B showed high correlation [89].

We also performed ELISA to estimate the levels of VEGF-B and PlGF in the vitreous of 42 patients with DR undergoing vitrectomy. OCT was used to estimate macular volume (MV) and central retinal thickness (CRT). The results showed elevated VEGF-B levels in these patients, which showed moderate ($p<0.05$) and robust ($p<0.01$) correlation with CRT and MV, respectively. PlGF, however, did not show any statistically significant correlation. Thus, VEGF-B targeting in these patients might offer beneficial therapeutic outcomes [90].

VEGF-B is expressed in the eye and its expression is upregulated after pathological challenge of the retina [48]. A recent study showed that subretinal injection of adeno-associated viruses encoding VEGF-B_{167} or VEGF-B_{186} increased ischemia and laser injury-induced retinal and choroidal neovascularization, respectively [86]. Another study showed that targeted inhibition of VEGF-B by shRNA (short hairpin RNA) or intravitreal injection of neutralizing antibody suppressed choroidal and retinal neovascularization in mice [43]. Thus, it can be concluded that VEGF-B targeting inhibited retinal neovascularization.

It appears that the ‘angiogenic’ activity of VEGF-B during ocular neovascularization is probably because of its potent survival effect on vascular and nonvascular cells. In addition,

both NRP-1 and VEGFR-1 participate in mediating the vascular survival effect of VEGF-B. Therefore, even though VEGF-B has a minimal role during the initial phase of blood vessel growth, the vascular survival activity of VEGF-B, which protects the neovessels from apoptosis may play a significant role in enhancing ocular neovascularization. Thus, targeted VEGF-B inhibition may also have therapeutic implications for the treatment of ocular neovascular diseases [43]. VEGF-B is currently receiving attention because of recent exciting advances in VEGF-B biology. Owing to its antiapoptotic and potent survival effect, and its ability to remain inactive under normal conditions, VEGF-B appears to possess valuable therapeutic potential for the treatment of degenerative diseases with an attractive safety profile.

3.3. PlGF

PlGF, originally isolated from the human placenta, participates in pro-angiogenic processes not only by direct signaling through VEGFR-1, but also indirectly by amplifying VEGF-A angiogenesis through regulation of the VEGFR-1 and VEGFR-2 cross-talk [91]. Although PlGF and VEGF-A are both expressed during neonatal retinal development, they have different modulatory influences on retinal vascular development [92]. Studies show that PlGF synergizes with VEGF-A for angiogenesis-associated eye diseases. Indeed, PlGF levels were elevated in the vitreous [93] and aqueous humor of patients with DR and NVG [94]. Another comparative study of vitreal PlGF levels in patients with proliferative DR with or without bevacizumab (anti-VEGF therapy) treatment showed that PlGF level was high in DR patients irrespective of the status of bevacizumab therapy and that it correlated strongly with VEGF-A levels. Thus, PlGF is implicated in DR pathogenesis in parallel to the involvement of VEGF-A, and use of aflibercept (anti-PlGF) might be beneficial in such cases [95]. Interestingly, PlGF deletion in a diabetic mouse model inhibited Akt signaling and HIF1 alpha-dependent VEGF-A activation, indicating that PlGF is required for VEGF-A-mediated DR pathogenesis

[96]. Furthermore, intraocular injection of PlGF gene or protein causes retinal vessel disorganization, dilatation, microaneurysm formation, retinal-blood barrier disruption, and edema [97].

Although high VEGF-A levels is one of the main risk factors for angiogenic eye diseases, more factors are currently indicated in the development of subretinal angiogenic pathogenesis. Consequently, Rakic et al. showed that PlGF levels were elevated in cases of choroidal neovascularization, whereas a VEGF-A isoform was present in the early stages of angiogenesis [98]. Huo et al. [99] used a laser burn mouse model of choroidal neovascularization to show that both PlGF and VEGF-A levels were elevated in mouse eyes and that these two factors coordinated to regulate choroidal neovascularization during ocular injury; anti-PlGF therapy alone did not stem the increase in vessel density post laser burn, but it augmented the anti-angiogenic function of anti-VEGF-A in their model.

We analyzed vitreous and serum PlGF levels in diabetic and non-diabetic patients undergoing vitrectomy (n=17 for diabetic and n=21 for non-diabetic) using ELISA. Results showed that vitreous PlGF levels were higher in the diabetes group and that it increased with severity of the disease, i.e., levels were higher in proliferative DR and in non-proliferative DR. Serum PlGF levels were also elevated in the diabetes group, although the mean difference was not statistically significant. In this work, we did not observe any correlation between vitreous and serum PIGF levels [100].

4. Targeting VEGF in retinal diseases

4.1. Current drugs

Ocular angiogenesis is a cause of severe visual loss. The treatment of ocular neovascular diseases is challenging and has improved dramatically in the last few years with the development of anti-VEGFs, which transformed the treatment of eye disorders. However, currently there is no cure, only therapies that slow down the progression of the disease.

According to a review by Tah and colleagues [101], anti-VEGF started its appearance approximately in 1948, however only after several years later the vascular permeability factor (VPF) was described and in 1989 it was named, as we know, VEGF.

Despite not being an angiogenic agent, one of the first pharmacological therapies to treat AMD was verteporfin (Visudyne®, Novartis Pharmaceuticals Corporation, East Hanover, New Jersey), an angioocclusive drug that in 2000 changed the course of AMD [102]. The aim of verteporfin therapy was to occlude vessels within the choroidal neovasculature while preserving the overlying retinal tissue.

A short time after, anti-angiogenic agents clinical trials began with powered outcomes of VEGF inhibition. VEGFs were recognized as angiogenic regulators of neovascularization and promoters of vascular permeability reasons for key targets for treating neovascular diseases. They become the standard care therapy for conditions involving neovascularization. The first phase I clinical trials in colon cancer with an anti-angiogenic drug were performed in 1997 by Genentech with the drug called bevacizumab (Avastin®, Genentech, Inc., San Francisco, California, USA), which was granted approval by U.S. Food and Drug Administration (FDA) in 2004 for the treatment of colon cancer as adjuvant to chemotherapy [102].

Additionally, in December 2004, FDA approved pegaptanib sodium (Macugen®, OSI Pharmaceuticals, Inc., Melville, New York) that was the first anti-VEGF therapy for neovascular AMD [102]. However, after bevacizumab approval for cancer therapy,

ophthalmologists began to use this molecule intravitreally to treat ocular neovascularization (off-label). Furthermore, Genentech designed a new molecule from the same precursor as bevacizumab, generating a different molecule, specifically for intravitreal use. This new molecule, ranibizumab (Lucentis®, Genentech Inc., San Francisco, California, USA) is believed to penetrate better into the retina [101]. Additionally, to decrease systemic adverse events, the portion Fc of the monoclonal antibody was removed from the original precursor. This new molecule showed to be safe and effective in the first clinical trials: MARINA (Minimally Classic/Occult Trial of the Anti-VEGF Antibody Ranibizumab in the Treatment of Neovascular Age-Related Macular Degeneration) and ANCHOR (Anti-VEGF Antibody for the Treatment of Predominantly Classic Choroidal Neovascularization in Age-Related Macular Degeneration) [102].

In November 2011, another anti-angiogenic agent, aflibercept (Eylea®, Regeneron Pharmaceuticals Inc., Tarrytown, New York) developed by Regeneron was approved by FDA [102] based on the VIEW studies, which showed to be a safe and an effective drug like ranibizumab. Currently, there are four therapies involving VEGF inhibition [103]:

1. Pegaptanib sodium intravitreal injections, (Macugen®), a pegylated VEGF aptamer. A single strand of nucleic acid that binds with specificity to the 165 isoform of VEGF-A was approved in 2004 by FDA for the treatment of neovascular AMD [102].

2. Bevacizumab (Avastin®) is a humanized anti-VEGF-A monoclonal IgG antibody developed as an anti-angiogenic agent for colon-rectal cancer, lung cancer, glioblastoma, and renal-cell carcinoma. It was approved for medical use in the United States in 2004 [102]. Bevacizumab has been used intravitreally (off-label) in the treatment of proliferative eye diseases.

3. Ranibizumab (Lucentis®) is a fully humanized monoclonal antibody fragment targeted against human VEGF-A, with high affinity for all isoforms of VEGF-A. Until the arrival of

ranibizumab, the primary endpoint in clinical trials was the proportion of subjects losing <15 letters. Ranibizumab changed the landmark of treatment with an unexpectable vision gains and a turnover of the clinical trials primary endpoint to the proportion of subjects gaining ≥ 15 letters. The binding of ranibizumab to VEGF-A at the receptor-binding site prevents the interaction of VEGF-A with its receptors VEGFR-1 and VEGFR-2 on the surface of the endothelial cells, inhibiting the cascade of events that leads to increased vascular permeability, increased activity and proliferation of endothelial cells and inflammation. Ranibizumab granted FDA approval in June 2006 [102].

4. Aflibercept (Eylea®) is a fusion protein of key domains from the human VEGFR-1 and VEGFR-2 and the human immunoglobulin G (IgG) Fc domain that was originally developed for oncology use. It binds to all isoforms of VEGF-A, to VEGF-B and PIGF. Aflibercept was approved for ocular use as Eylea®, and for metastatic colorectal cancer as Zaltrap® [102]. Eylea® was approved in 2011 by the FDA.

VEGF inhibitors are promising drugs in the treatment of neovascular eye diseases, however, should be of noted that there are some limitations of its usage [103]:

1. The unknown duration of anti-neovascular effects. Anti-angiogenics reduce regression of neovascularization after treatment and improve structural and functional parameters, but the duration of the effect is limited to a short period of time.

2. The mode of drug delivery, an intravitreal injection. There are complications after intravitreal injections, such as endophthalmitis, intraocular inflammation, rhegmatogenous retinal detachment, acute intraocular pressure elevation and ocular hemorrhage.

3. Anti-VEGF therapy requires frequent injections and assessments to determine patient response to a treatment. This fact leads to a significant burden of injections and visits for all involved in the treatment of those patients. The solution for this problem would be a

medication that improves visual and structural outcomes, simultaneously increasing drug effectiveness and lengthening the durability of the treatment.

4. The frequent anti-angiogenic therapy may be associated with the progression of geographic atrophy.

5. Another issue is the systemic safety. Several pharmacovigilance reports have been generated with the administration of either systemic or ocular anti-VEGFs: thromboembolic events, myocardial infarction, stroke, hypertension, gastrointestinal perforations, and kidney disease which lead to the inclusion of a black box in the summary of product characteristics. Moreover, intravitreal antiangiogenic drugs were found at detectable levels in the systemic circulation, capable to suppress VEGF-A systemic levels. This fact suggests a rationale for the cardiovascular reports of serious adverse events. Although the death rates did not seem to be increased due to the use of angiogenic drugs, the long-term consequences are still unknown.

Furthermore, VEGF-A, VEGF-B, and PIGF play critical roles in neuroprotection and cardioprotection [103]. Therefore, their blockage may also have consequences in the long run. Nevertheless, not all patients respond sufficiently to anti-VEGF intravitreal injections despite frequent treatments. About 50% of patients have an insufficient response to angiogenic therapy and accordingly with an analysis performed by Gonzalez and colleagues, a percentage of patients would benefit from an early therapy switch as shown in the EARLY study [104]. Therefore, and despite not being an angiogenic therapy, corticosteroids treatments are invaluable and one of the oldest treatments available in ophthalmology for the treatment of persistent or recurrent diseases. Steroids have proven to be powerful and effective in suppressing inflammation and also playing a significant role in inhibition of several cytokines inclusively antagonizing the action of VEGF-A.

The best-studied steroids are triamcinolone acetonide (off-label), dexamethasone, and fluocinolone. Dexamethasone implant (Ozurdex®, Allergan, Dublin, Republic of Ireland) is a bioerodable, extended-release of 700 μg of dexamethasone in a solid, bioerodable polymer [84]. Fluocinolone acetonide (Iluvien®, Alimera Sciences, Alpharetta, Georgia) is the smallest, non-bioerodable, slow extended-drug release implant lasting 3 years of duration [84]. Notwithstanding the well-known side effects caused by steroids, cataract formation and increase of intraocular pressure, efficacy and benefits usually outweighed risks. Moreover, there is an enormous advantage of corticosteroids once systemic side effects of locally administered steroids occur rarely. Although laser photocoagulation, anti-VEGFs and steroids pathways established as successful target treatments, new therapeutical enhancements are being developed, holding promises in the improvement of eye pathologies.

4.2. Drug Discovery – searching for promising molecules

Several diseases are accompanied by dysregulated angiogenesis and by excessive formation of blood vessels, such as in cancer, RA, AMD, RVO, and PDR or by deficiency in blood vessels, such as in heart and limb ischemia. These diseases can be life-threatening as they cause severe pain and reduce the quality of life. They are also considered to be a burden to the society, not only because of costs incurred but also due to the time-consumed by the physicians, patients, and respective families.

It is therefore of great importance with regard to improve and develop better treatments. The VEGF family and the anti-angiogenics are fascinating molecules that had been receiving interest and research efforts all over the world. Researchers and pharmaceutical companies have been involved in the search for the critical need for new therapies to be given early on in the disease to cure or delay its progression or to prevent. However, there is still a lack of therapeutically viable options for intervention.

Regardless, a remarkable selection of compounds has been described in recent years, targeting different molecules in the signaling pathway, from VEGF-A, VEGF-B, PIGF, and platelet derived growth factor (PDGF), and tyrosine kinase inhibitors (TKIs) to VEGFRs and tyrosine kinase receptor (TKR) inhibitors (Figure 2). These compounds comprise not only the above described pegaptanib, bevacizumab, ranibizumab, and aflibercept, but also other new molecules such as abicipar pegol, various siRNA, avancincaptad pegol, brolucizumab, multi VEGF-PDGF DARPin, TB-403, and 5D11D4 and TKIs such as lapatinib, sunitinib, sorafenib, axitinib, and pazopanib. Some of those drugs are approved and are used against retinal diseases; however, others are in the clinical or pre-clinical stage. Table 1 summarizes the anti-angiogenic drugs currently approved and available, the molecules on pre and post clinical trials, and possible drugs never studied in ophthalmology that may be considered in the future as targeted molecules.

Significant progress has been made in the understanding of the molecular pathogenesis of retinal neovascular disorders and new targets have been investigated for therapeutic interventions.

5. Outcome and future perspectives

We reviewed literature regarding the involvement of VEGF growth factors, especially VEGF-A, VEGF-B, and PlGF in normal development and diseases with special emphasis on ocular disorders. We showed that accurate estimation of vitreous/aqueous and serum levels of these factors are critical for elucidating their roles in ocular disease pathogenesis. Studies are mainly focused on the involvement of vitreous and serum VEGF-A levels with the prognosis of AMD, DR, and RVO, whereas the role of VEGF-B and PlGF as biomarkers for these conditions is beginning to be understood (Table 2). Table 2 shows that elevated vitreous and/or serum levels of VEGF-A were detected in patients with different ocular disorders, and the levels increased with the severity of the disease [108,110]. The angiogenic potential of

VEGF-A aggravates the pathology of the diseases and anti-VEGF-A therapy is often beneficial for ameliorating or regressing the symptoms. However, certain cases are also characterized by high VEGF-B and PlGF levels in the vitreous of the diseased eye. Our work and few other studies have revealed the importance of screening patient samples for VEGF-B and PlGF levels as future prognostic markers or targeted therapies [88-90,100]. Reports show that PlGF often synergizes VEGF-A signaling in retinal pathologies and therefore, targeting PlGF should be critical for completely containing the symptoms [62,66]. Similarly, VEGF-B is an emerging druggable candidate for the treatment of angiogenic ocular disorders [59]. Finally, caution should be exercised while interpreting the vitreous and serum levels of these growth factors as high vitreous levels may sometimes be due to increased vascular permeability, which allows serum proteins to leak inside the vitreous and result in false positive results. [124]. Contrary to this, some authors describe that vitreous levels are independent of serum levels as it is not easy to find a positive and robust correlation between them [119]. Further, caution must be exercised during interpretation of serum levels and growth factors as other systemic diseases may increase VEGF levels. Therefore, robust correlation between vitreous and serum levels of these growth factors would increase the confidence level of the approach.

Multiple challenges in the treatment of ocular diseases include: 1) identification of biomarkers before diagnosis and appearance of clinical symptoms, which would enable treatment of early stages of the diseases, 2) identification of markers to monitor disease progression, and 3) identification of new targeted therapies. More clinical trials in ophthalmology are required to test the efficacy and safety of new therapies targeting VEGF-B and/or PIGF, either in combination with existing therapies or in monotherapy.

Future research should continue to focus on new anti-VEGF strategies in the treatment of ocular diseases linked to abnormal vascularization. Discovery of new isoforms of the VEGF

family reveal an increased biological complexity and has faced many obstacles that must be overcome while exploring new targets. The blockage selectivity is one of the most important factors to be considered when testing the new anti-VEGF molecules.

Understanding the detailed molecular mechanisms underlying angiogenesis and physiology is of vital importance for the future development of drugs, which can regulate angiogenesis. However, there are many unanswered questions that need to be explored as well as new and innovative molecules to be discovered before the full potential of these strategies can be understood, which would result in better outcomes for patients with pathological ocular angiogenesis.

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Conflicts of interest

None of the authors have any conflicts of interest to declare.

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Table 1. Summary of anti-angiogenic agents [105,106, 107]

Generic drug name	Trade name	Type of molecule	Target	Clinical stage in ophthalmology	Therapeutic Indications	Route of administration	Commercialized by
Pegaptanib	Macugen®	RNA aptamer	VEGF-A $_{165}$	Commercialized	Wet AMD	Intravitreal injection	OSI Pharmaceuticals/Pfizer / Bausch & Lomb
Bevacizumab	Avastin®	Recombinant humanized full monoclonal antibody	All VEGF-A isoforms	Not commercialized for ocular use	Off-label usage	Intravitreal injection	Genentech in U.S. and Roche in Europe
Ranibizumab	Lucentis®	Recombinant humanized monoclonal antibody fragment	All VEGF-A isoforms	Commercialized	Wet AMD, macular edema following RVO, DME, DR with DME, and myopic CNV	Intravitreal injection	Genentech in U.S. and Novartis in Europe
Aflibercept	Eylea®	Fusion protein	All VEGF-A & VEGF-B isoforms and PIGF	Commercialized	Wet AMD; ME following RVO, DME and DR in Patients with DME	Intravitreal injection	Regeneron in U.S. and Bayer in Europe
Conbercept/ KH902	Lumitin	Fc fusion protein	All VEGF-A & VEGF-B isoforms, VEGF-C and PIGF	Commercialized in China	Wet AMD	Intravitreal injection	Chengdu Kanghong Biotechnology Co.
AGN-150998; Abicipar Pegol	-	DARPin	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Allergan
E10030; Pegplenarib	Fovista®	DNA aptamer	PDGF-BB	Phase III	Wet AMD	Intravitreal injection	Ophthotech/ Novartis
ESBA-1008; RTH258 (brolucizumab)	-	Humanized single chain anti-body fragment	VEGF-A	Phase III	Wet AMD, DME	Intravitreal injection	Alcon Research
TB-403; THR 317	-	Monoclonal antibody	PIGF	Phase II – DME Pre-clinical - DR	DME, DR	Intravenous infusion	ThromboGenics /Roche
Sunitinib maleate-	Sutent® (oral)	TKI	Small molecules	_	**	Intravitreal	GrayBug Inc.
Sorafenib	Nexavar®	TKI	Small molecules	Phase I/II AMD	Hepatocellular and Renal cell carcinoma; Thyroid cancer	Oral	Bayer
Axitinib/ Axitinib ophthalmic	Inlyta® Oral formulation /Pfizer)	TKI, PDGF, VEGF-A	Small molecules	Preclinical DME, RVO	Renal cell carcinoma	Oral	Clearside Biomedical

Note: CNV – Choroidal neovascularization, DARPin - Designed Ankyrin Repeat Proteins, DDIT4 mRNA- DNA-damage-inducible transcript 4 mRNA, DME – diabetic macular edema, DR – diabetic retinopathy, KSP mRNAs - Kinesin spindle protein mRNAs, PDGF - Platelet-derived growth factor, PIGF - placental growth factor, RVO – retinal vein occlusion, siRNA– Small interfering RNA, VEGF - vascular endothelial growth factor, VEGFR-1 - vascular endothelial growth factor receptor 1, Wet AMD – Wet age related macular degeneration. TKI- tyrosine kinase inhibitors; ** Sunitinib licensed for Pfizer is marketed for gastrointestinal stromal tumors; pancreatic cancer; renal cell carcinoma.

Table 2. Comparative list of VEGF-A, VEGF-B, and PlGF levels in vitreous, aqueous and serum samples of patients with various ocular diseases

Number of patients	Mean vitreous/aqueous VEGF-A/VEGF-B/PlGF levels (study and control)	Mean serum VEGF-A/VEGF-B/PlGF levels study and control	Comments of significance	Reference
57 PDR vs 16 controls vs 6 NPDR	1135.2 pg/mL VEGF-A vs 19.3 pg/mL VEGF-A ($p<0.0001$) vs 49.9 pg/ml VEGF-A ($p<0.0001$).	ND	Vitreous levels of VEGF-A were significantly higher in patients with active PDR than in those with quiescent PDR ($p<0.0001$).	[108]
20 PDR vs 20 controls	1340 pg/mL VEGF-A vs 9 pg/mL VEGF-A (median) ($p<0.0001$).	177 pg/mL VEGF-A vs 170 pg/mL VEGF-A (median), (no signficant difference).	Direct correlation between VCAM-1 and VEGF-A, suggest connection between cellular adhesion and neovascularization.	[109]
11 PDR vs 23 control	7200 pg/mL VEGF-A vs 1800 pg/mL VEGF-A ($p<0.001$).	ND	Vitreous VEGF-A levels are associated with DR. VEGF-A plays a critical role in the DR progression.	[110]
23 PDR vs 17 controls	1420 pg/mL VEGF-A vs 9 pg/mLVEGF-A ($p<0.001$).	120 pg/mL VEGF-A vs 150 pg/mL VEGF-A (ns).	VEGF-A is increased in the vitreous of diabetic patients with PDR, suggesting intraocular production as the main factor for the intravitreous enhancement of VEGF-A.	[111]
41 PDR vs 18 controls	812 ± 1.108 pg/ml VEGF-A vs 1.7 ± 4.4 pg/mL VEGF-A ($p<0.0001$).	ND	VEGF-A levels in eyes with active PDR were significantly higher than in those with inactive PDR.	[112]
39 PDR vs 11 controls	1134 pg/mL VEGF-A vs <50 pg/mL VEGF-A ($p<0.001$), (median values).	ND	Controls and patients without PDR had low and comparable VEGF-A levels (medians < 50 pg/mL). Patients with PDR had high vitreous VEGF-A concentrations (median 1134 pg/mL).	[113]
16 NPDR vs 13 controls	192.7 pg/mL VEGF-A vs < 31.2 pg/mL VEGF-A (median values), ($p<0.001$).	414.3 pg/mLVEGF-Avs332.7pg/mL VEGF-A (ns).	No significant correlations were found between concentrations of VEGF-A in serum and vitreous.	[114]
37 PDR vs 21 controls	1380 pg/mL VEGF-A vs 9 pg/mLVEGF-A ($p<0.0001$)	130 pg/mL VEGF-A vs 160 pg/mL VEGF-A.	No correlation between serum and vitreous in diabetic patients nor in control group ($r=0.06$, p=ns and $r=-0.15$, p=ns, respectively)	[72]
70 PDR vs 25 NPDR vs 41 quiescent PDR vs 31 controls	3600 ± 6300 pg/mL VEGF-A vs 100 ± 100 pg/mL VEGF-A ($p=0.008$) vs 200 ± 600 pg/mL VEGF-A ($p<0.001$) vs 100 ± 200 pg/mL VEGF-A ($p=0.003$).	ND	VEGF have an important role in mediation active intraocular neovascularization in patients with ischemic retinal diseases, such as DR and RVO.	[69]
27 PDR vs 14 controls	410 pg/mL VEGF-A vs 17 pg/mL VEGF-A (median values), ($p<0.001$).	VEGF-A levels in PDR (median values) 190 pg/ml lower than vitreous VEGF-A ($p<0.05$).	Vitreous levels of VEGF-A were higher in PDR, and not influenced by its serum concentrations. VEGF-A plays an important role in neovascularization of PDR.	[115]
22 PDR vs 28 controls	1759 ± 1721 pg/mL VEGF-A vs 27 ± 65 pg/mL VEGF-A ($p<0.001$).	ND	Correlation of glutamate and GABA levels with high VEGF-A levels providing biochemical support for ischemia-induced neovascularization in PDR.	[116]
19 PDR vs 7 controls	5660 pg/mL VEGF-A vs 350 pg/mL	ND	VEGF-A vitreous levels in PDR were elevated, and play a role	[117]

	VEGF-A ($p<0.05$).		in its pathogenesis.	
30 PDR vs 35 controls	383.1 ± 107.48 pg/mL VEGF-A vs 24.81 ± 1.85 pg/mL VEGF-A ($p\leq0.005$) respectively.	515.12 ± 44.8 pg/mL VEGF-A vs 343.58 ± 46.41 pg/mL VEGF-A ($p\leq0.005$), respectively.	Positive correlation between serum and vitreous VEGF-A levels ($p=0.012$ and $r=0.453$).	[118]
20 PDR vs 13 controls	1.75ng/mL VEGF-A vs 0.009 ng/mL VEGF-A (median values).	No differences in serum VEGF-A between groups.	High vitreous VEGF-A levels in PDR patients were not attributed to serum levels.	[119]
20 PDR vs 12 controls (MH)	833.7 ± 281.3 pg/mL VEGF-A vs 32.9 ± 18.1 pg/mL VEGF-A ($p>0.001$).	30.2 ± 11.8 pg/mL VEGF-A vs 22.1 ± 9.2 pg/mL VEGF-A ($p>0.05$), respectively.	Vitreous VEGF-A significantly higher vs serum in diabetic patients.	[120]
46 PDR vs 49 NPDR vs 31 without DR vs 28 healthy subjects	ND	149.12 pg/mL VEGF-A vs 153.07 pg/mL VEGF-A vs 125.37 pg/mL VEGF-A vs 98.20 pg/mL (median values).	No correlation between macular thickness and serum VEGF-A levels ($p>0.05$).	[121]
31 PDR vs 15 controls	Vitreous VEGF-A significantly increased in the PDR vs controls.	No differences were evident in serum VEGF-A.	No correlation between the vitreous and serum levels of VEGF in patients with PDR.	[122]
42 PDR vs 48 without DR	ND	219 ± 99 pg/mL VEGF-A vs 139 ± 98 pg/mL VEGF-A ($p<0.001$).	Serum VEGF-A was significantly higher in patients with PDR than in those without DR.	[123]
15 PDR vs 15 NPDR vs. no DR vs 15 normal controls	ND	616 ± 301 pg/mL VEGF-A ($p<0.05$) vs 787 ± 476 pg/mL VEGF-A ($p<0.05$) vs 508 ± 262 pg/mL VEGF-A vs 323 ± 8 pg/mL VEGF-A.	No correlation between serum VEGF-A and severity of DR. Serum VEGF-A levels in NPDR are higher than in PDR patients. Circulating VEGF-A is involved in progression of DR.	[124]
45 PDR vs 28 controls	723.21 pg/mL VEGF-A vs 20.81 pg/mL VEGF-A respectively ($p<0.001$).	ND	Vitreous VEGF-A significantly higher in active PDR than in eyes with inactive PDR ($p=0.008$).	[125]
20 samples (PDR and controls)	Vitreous VEGF-A levels were significantly higher in eyes with PDR than in eyes without PDR ($p=0.006$).	ND	VEGF-A functions as a physiologically angiogenic factor in PDR.	[126]
22 NVG patient vs20 controls (aqueous humor)	3037 ± 2387pg/mL VEGF-A in NVG patients 1078 ± 712 pg/mL PlGF ($p<0.001$ with respect to control) in NVG patients.	Serum levels of VEGF-A and PlGF were low in the patients and controls.	High concentrations of VEGF-A correlated with high levels of PlGF in patients with NVG ($r = 0.593$, $p = 0.004$). Concentrations of VEGF-B in aqueous humour and serum remained unchanged ($p>0.05$). Positive correlation between VEGF-A and PlGF aqueous humor.	[127]
30 NVG vs 30 control eyes (aqueous humor)	832.88 ± 96.44 pg/mL VEGF-A vs 206.5 ± 45.84 pg/mL VEGF-A.	356.88 ± 68.45 pg/mL VEGF-A vs 112.54 ± 65.13 pg/mL VEGF-A.	Aqueous VEGF-A significantly different between patients and control ($p<0.001$). Positive correlation between serum and aqueous VEGF-A levels in the NVG group ($r=0.638$, $p=0.001$).	[128]

71 patients with DME	VEGF-A in the pre-macular vitreous (1386.2 ± 2134.1pg/mL) vs the peripheral cortical vitreous (1169.7 ± 1840.3 pg/mL, p=0.0216) vs mid-vitreous (1080.9 ± 1534.1 pg/mL; p=0.0017). Controls VEGF-A concentrations in the premacular vitreous, peripheral cortical vitreous and mid-vitreous were all below the detection limit (<20 pg/mL).	ND	Vitreous VEGF-A concentration followed a gradient. VEGF-A was higher in premacular vitreous than in mid-vitreous and peripheral cortical vitreous, suggesting diffusion from the macular region to the periphery, and from the posterior to the anterior globe.	[82]
37 diabetic vs 8 controls	Mean PIGF levels in diabetics 103 pg/mL vs non-detectable in control samples.	ND	PIGF was present in all diabetic vitreous samples but non-detectable in controls. The results demonstrated a role for PIGF in the pathogenesis of PDR.	[129]
10 IR vs 26 controls (aqueous humor) plus vitreous samples from: 11 eyes with PDR, 2 eyes with non-diabetic IR (central RVO, acute retinal necrosis) and 7 eyes without IR	PIGF was detected in 1 out of 36 aqueous samples with severe PDR at a very high concentration (2270 pg/mL). Vitreous PIGF concentration (n=13) from 11 eyes with PDR was 360 ± 272 pg/mL. PIGF concentration in the 2 eyes with non-diabetic IR was 458 pg/mL. PIGF not detected in the eyes without IR.	ND	The concentration of PIGF was significantly correlated with that of VEGF-A (n=19, r=0.526, p=0.019)	[130]
50 PDR vs 19 control	Vitreous PlGF and vitreous VEGF-A levels (median range) in PDR (PlGF, 100.6 pg/mL, range 7.6 – 1,038.6; VEGF-A 653.9 pg/mL, 9.0 – 5, 423.8) were significantly higher (p>0.0001) than in the control (PlGF 7.0 pg/mL, 7.0 – 12.1; VEGF-A 9.0 pg/mL, 9.0 – 10.0).	ND	The ratio of vitreous PlGF and vitreous VEGF-A to protein in active PDR was significantly higher than that in quiescent PDR (PlGF 33.5, 2.7–250.7 vs 11.1, 1.5–35.8, p=0.0039; VEGF-A 130.1, 7.8–904.0 vs 73.9, 2.0–150.3, p= 0.0328). Intravitreous PlGF levels significantly correlated with intravitreous VEGF levels in both PDR patients (r = 0.824, p< 0.0001) and total subjects (r = 0.857, p< 0.0001).	[92]
50 PDR vs 56 controls	Vitreous VEGF-A was 585.67 ± 57.40 pg/mL in the PDR vs 123.85 ± 109.42 pg/mL in controls.	VEGF-A concentration (plasma) was 410.07 ± 74.70 pg/mL in the PDR vs 114.41 ± 110.99 pg/mL in healthy controls.	Vitreous and plasma VEGF-A levels were significantly elevated in PDR patients than those in controls (p vitreous<0.001, p plasma<0.001). Both vitreous and plasma VEGF-A levels were significantly higher in PDR progression group than in stable group (p vitreous < 0.001; p plasma = 0.004). Vitreous VEGF-A was positively associated with plasma VEGF-A in PDR patients (p<0.001).	[131]
20 PDR (DMI) vs 16 PDR (DMII) vs control	Vitreous VEGF-A in diabetics with PDR and DMI was 432.23 pg/mL, in diabetics with PDR and DMII was 147.5 pg/mL and in controls was 63.26 pg/mL (p<0.005).	ND	Mean vitreous VEGF-A levels of diabetic patients were significantly higher than in controls. Mean vitreous VEGF-A levels were significantly higher in diabetics with PDR and DMI than in diabetics with PDR and DMII.	[132]
25 diabetic patients with PDR (n =19) or NPDR (n = 6) vs control group	Vitreous VEGF- B was higher in the diabetic group (18.82 ± 1.44 pg/mL) vs control group (17.90 ± 0.32pg/mL), (p =	ND	Mean vitreous VEGF- B was higher in PDR (19.03 ± 1.52pg/mL) vs NPDR (18.18± 0.96pg/mL). VEGF-B was significantly increased in DR, and this increase is significantly	[88]

of 8 non-diabetic patients	0.006).		higher as the DR is at a more advanced stage.	
21 diabetic (4 NPDR and 17 PDR) vs 17 non-diabetic	Mean vitreous PIGF was 70.0 vs 46.47 pg/ml, Z =-2,847, p = 0.004.	Mean serum PIGF was 50.5 vs 48.8 pg/mL (Z = -1,196, p= 0.232).	PDR patients had significantly elevated vitreous PIGF vs. NPDR patients, (76.5 vs. 42.5 pg/ml, Z=-2.612, p=0.009). PIGF is overexpressed in the vitreous of diabetic patients and levels increase with the severity of the disease. No correlation between vitreous and serum levels of PIGF.	[100]
14 patients with POD: 8 DR, 1 RVO, 1 AMD vs 4 NPOD patients with VMTS vitreomacular traction syndrome	Mean vitreous VEGF-A and B was 603.65 ± 688.60 and 368.46 ± 451.46 pg/mL in POD, respectively vs non-detectable values in NPOD.	Mean serum VEGF-A and B in POD was 101.28 ± 60.68 and 53.72 ± 42.39 pg/mL vs 81.82 ± 64.97 (p=0.604) and 41.55 ± 35.62 pg/mL (p=0.777) in NPOD.	There was a strong and positive statistically significant correlation between VEGF-A and B in vitreous and serum samples.	[89]

Note: DMI: Diabetes mellitus I, DMII - Diabetes mellitus II, DR - Diabetic retinopathy, IR – Ischaemic retinopathy, ND – not done, NPDR - Non-PDR, ns – not statistical significant, MH - Macular Hole, NVG – Neovascular glaucoma, POD - proliferative ocular disease, PDR - Proliferative diabetic retinopathy, RVO – retinal vein occlusion, VCAM-1 – vascular cell adhesion molecule 1, VEGF – vascular endothelial growth factor, VMTS - vitreomacular traction syndrome. Results are provided as mean ± SD (standard deviation) when available.

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Figure Captions

Figure 1 – Factors involved in upregulation of VEGF-A and main repercussions. Hypoxia is one the most important through HIF-1, followed by hyperglycemia; AGEs and pro-inflammatory cytokines are other stimulating factors that increase VEGF-A production. The main consequences for retinal diseases are the BRB breakdown with increasing vascular permeability and neovascularization.

AGEs – Advanced glycation end products; BRB – Blood retinal barrier; HIF-1α – Hypoxia-inducible factor 1-alpha; IGF-1 – Insulin –like growth factor 1; FGF – Fibroblast growth factor; PDGF – Platelet-derived growth factor

Figure 2 - The VEGF family, receptors and current anti-angiogenic drugs that target VEGF/VEGFR signalling.